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WITNESS my hand this
Ninth day of September 2003

J R Yabsley

JONNE YABSLEY
TEAM LEADER EXAMINATION
SUPPORT AND SALES

P/00/009
Regulation 3.2

AUSTRALIA
Patents Act 1990

PROVISIONAL SPECIFICATION

Invention Title: METHODS OF REGULATING DIFFERENTIATION IN STEM CELLS

Applicant: ES CELL INTERNATIONAL PTE LTD

The invention is described in the following statement:

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METHODS OF REGULATING DIFFERENTIATION IN STEM CELLS

FIELD

The present invention relates to the culture of stem cells. More specifically the present invention relates to methods and compositions for inhibiting differentiation of stem cells in culture. Also provided are pharmaceutical compositions and methods of treatment related to disorders of cell differentiation.

BACKGROUND

In general, stem cells are undifferentiated cells which can give rise to a succession of mature functional cells. For example, a haematopoietic stem cell may give rise to any of the different types of terminally differentiated blood cells. Embryonic stem (ES) cells are derived from the embryo and are pluripotent, thus possessing the capability of developing into any organ, cell type or tissue type or, at least potentially, into a complete embryo. ES cells may be derived from the inner cell mass of the blastocyst, which have the ability to differentiate into tissues representative of the three embryonic germ layers (mesoderm, ectoderm, endoderm), and into the extra-embryonic tissues that support development.

Because of their ability to differentiate into any cell type, the ability to culture ES cells *in vitro* is clearly desirable. However a significant problem is that ES cells tend to spontaneously differentiate in culture, leading to a loss in their pluripotency. This is undesirable as it would be beneficial to expand a population of hES cells without a loss in pluripotency.

This problem is conventionally addressed by using mammalian serum in culture systems for hES cells, and it has been shown that the removal of serum from ES cultures leads to a rapid loss of stem cell phenotype. However, serum contains a wide variety of biologically active substances which have the potential to adversely effect ES cell growth and differentiation. Furthermore, there is a biosafety issue if cells cultured in animal serum are subsequently used for implantation in a human or for the production of a biological

therapeutic.

Other studies have employed supplemented defined media supplemented to culture hES cells. However differentiation of ES cells under using such media is still significantly more rapid than in the presence of serum, and animal cells are still required to maintain the hES cells.

Identification of the factors in serum responsible for inhibition of differentiation could facilitate design of simple culture media more suitable for human ES cell propagation. The prior art has not provided a satisfactory replacement for serum that allows for the culture of hES cells while inhibiting the differentiation of the cells

It is therefore an object of the present invention to at least alleviate a problem of the prior art.

The discussion of documents, acts, materials, devices, articles and the like is included in this specification solely for the purpose of providing a context for the present invention. It is not suggested or represented that any or all of these matters formed part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed in Australia before the priority date of each claim of this application.

SUMMARY OF THE INVENTION

The present invention provides a method for modulating differentiation of a stem cell having an Edg receptor, the method including the step of exposing the cell to an agonist of the Edg receptor.

Applicants have found that agonists of the Edg receptor such as sphingosine-1-phosphate (S1P), lysophosphatidic acid (LPA), platelet activating factor (PAF) and sphingophosphorylcholine (SPC) have the ability to at least partially inhibit the spontaneous loss of stem cell phenotype in cell culture. It has also been

found that the method does not lead to a substantial negative alteration in the ability of stem cells to proliferate.

The present invention also provides a cell culture medium substantially free of serum, the medium including an agonist of an Edg receptor.

The present invention further provides a cell grown and/or maintained in a cell culture medium substantially free of serum, the medium including an agonist of the Edg receptor.

Also provided by the present invention is a method for modulating an endothelial differentiation gene receptor, the method including the step of exposing a stem cell having the receptor to an agonist of the receptor.

The present invention also provides a method for modulating the MAP kinase pathway, the method including the step of exposing a stem cell having the pathway to an agonist of the Edg receptor.

Also provided by the present invention is a method for modulating the differentiation of a stem cell having a MAP kinase pathway and/or an endothelial gene receptor, the method including modulation of the MAP kinase pathway and/or endothelial gene receptor.

Another aspect of the present invention is a method of treating or preventing a disorder of stem cell differentiation including administering to an animal in need thereof a composition containing an agonist of an Edg receptor. Also provided are pharmaceutical compositions including compounds of the present invention

DESCRIPTION OF THE FIGURES

FIGURE 1 shows S1P inhibits the spontaneous differentiation of HES cells. (A) HES cells grown with feeder, before the depletion in serum. (B) HES cells grown without serum after 8 days (B, C) and 12 days (D, E), in absence (B, D) or presence of S1P (C, E, 10 μM). These data are representative of at least 3 independent experiments.

FIGURE 2 shows S1P inhibits the spontaneous differentiation of HES cells. Double staining experiments were performed using antibodies for PCNA and GCTM-2. These data are representative of at least 3 independent experiments.

FIGURE 3 shows S1P inhibits the spontaneous differentiation of HES cells. Double staining experiments were performed using antibodies for PCNA and GCTM-2. These data are representative of at least 3 independent experiments.

FIGURE 3 shows S1P stimulates ERKs phosphorylation in HES cells. Western blots experiments were performed using protein lysate from HES cells. (A) Cells were pre-treated or not with U0126 (30 μM, 1 hr) and incubated for 5 min in the absence (C, control) or presence of S1P (10 μM). (B) Cells were incubated for different time periods in the absence or presence of S1P (10 μM). (C) Cells were incubated for 5 min with various concentrations S1P. The phosphorylation of Erk1 and Erk2 (P-Erk1 and P-Erk2) was assessed by immunoblotting with a polyclonal anti-active MAP kinase as described in Materials and Methods. After a stripping procedure, the same blots reprobed with a monoclonal anti-MAP kinase, allowed the detection of Erk1 and Erk2. These data are representative of at least 3 independent experiments.

DETAILED DESCRIPTION OF THE INVENTION

In one aspect, the present invention provides a method for modulating differentiation of a stem cell having an Edg receptor, the method including the step of exposing the cell to an agonist of the Edg receptor. Applicants have discovered that binding of an agonist to the Edg receptor at least partially inhibits differentiation of the stem cell. In a preferred form of the invention the stem cell is an embryonic stem cell. More preferably the stem cell is a human embryonic stem cell.

Preferably, the endothelial differentiation gene receptor is selected from the group including Edg-1, Edg-3, and Edg-5.

As used herein the term "modulating the differentiation of a stem cell" includes the inhibition or enhancement of cellular differentiation. The term also includes partial inhibition or enhancement of cellular differentiation. In a preferred form of the method, the modulation is inhibition of differentiation.

In a preferred method, the stem cell is an embryonic stem cell. More preferably the stem cell is a human embryonic stem cell. As used herein the term "embryonic stem cell" means a cultured cell line derived from preimplantation stages of development capable of differentiation into tissues representative of all three embryonic germ layers.

Theses cells express SSEA-3,SSEA-4, TRA 1-60, GCTM-2, alkaline phosphatase, Oct-4

- Grow as flat colonies with distinct cell borders
- Differentiate into derivatives of all three embryonic germ layers
- Feeder cell dependent (feeder cell effect on growth not reconstituted by conditioned medium from feeder cells or by feeder cell extracellular matrix)
- Highly sensitive to dissociation to single cells, poor cloning efficiency even on a feeder cell layer
- Do not respond to Leukemia Inhibitory Factor

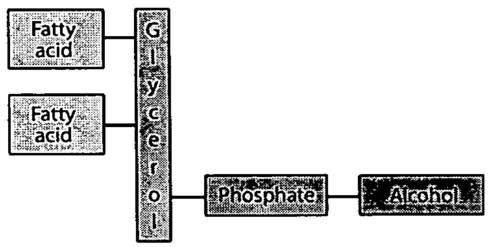
Preferably the agonist is selected from the group consisting of S1P, LPA, PAF and SPC.

Preferably the agonist is a phospholipid, lysophospholipid or functional equivalent thereof. Applicants have found that members of this class of compounds can act as agonists of Edg receptors on stem cells. Accordingly, by exposing a stem cell to a phospholipid or lysophospholipid the differentiation of stem cells can be at least partially inhibited

The bioactive effects of some phospholipids appear to be mediated by specific cell surface G-protein coupled receptors (GPCRs) called endothelial differentiation gene (Edg) receptors. To date, eight distinct mammalian Edg receptors have been identified Some of these receptors are widely expressed

(Edg-1, Edg-2, Edg-3, Edg-5 and Edg-7) while the others show a more restricted distribution (Edg-4, Edg-6 and Edg-8).

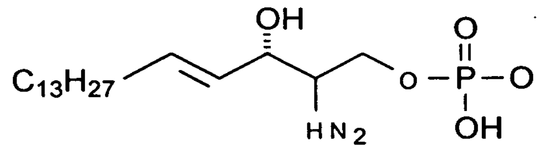
As used herein, the term "phospholipid" includes any molecule that includes a backbone attached to two fatty acid moieties and a phosphate group. The backbone on which the phospholipid is variable and may be based on glycerol or sphingosine for example. A diagram of a generic phospholipid is shown below.



The term "lysophospholipid" refers to a phospholipid molecule where one of the fatty acid chains has been removed. The removal of a fatty acid chain may be accomplished by treatment of the phospholipid with an enzyme such as phospholipidase A2.

Phospholipids and lysophospholipids of the present invention include but are not limited to cardiolipins, phosphatidylserines, lysophosphatidylserines, phosphatidylcholines, lysophosphatidylcholines, (including diacyl, γ-O-Alkyl, Di-O-Alkyl and β-Acyl-γ-O-alkyl forms) phosphatidylethanolamines, lysophopatidylethanolamines, phosphatidylinositols, lysophophatidylinositols, phosphotidylglycerols, lysophosphatidylglycerols, diphosphatidyl glycerol, sphingosine, sphingosine-1-phosphate.

In a preferred method of the invention the phospholipid or lysophoholipid has a sphingosine backbone. More preferably, the lysophospholipid is a phosphorlyated amino alcohol. In a highly preferred form of the invention the lysophospholipid is sphingosine-1-phosphate (S1P) or functional equivalent thereof. S1P is a small bioactive phospholipid, present in serum, released by activated platelets, which has the following structure:



The skilled person will understand that bioactive molecules such as phospholipids and lysophospholipids may be altered in a number of ways and still retain biological activity.. Accordingly, the scope of the present invention includes altered forms of phospholipids and lysopholipids. For example, the length of the fatty acid moieties may be altered and the resultant molecule may still be useful in the context of the present invention. Of course, some alterations may be introduced that will result in a significantly large structural change so that the lipid does not bind to its receptor. In addition, some changes may result in the compound having a higher affinity for the receptor. Other alterations include methylation, alkylation, acylation, esterification, phosphorylation and the like.

The skilled person will be familiar with methods for testing any phospholipid or lysophospholipid for the ability to modulate the ability of a human embryonic stem cell to differentiate. Suitable methods are found herein, and include reactivity with antibodies such as GCTM-2 which are directed to stem cell specific markers, and simple morphological evaluation of cells by light microscopy.

For example the effect of the agonist on the differentiation of stem cells into neuronal or endodermal lineages may be studies by analysis of marker expression as shown in PCT AU01/00278 and AU01/00735.

The phospholipid or lysophospholipid may be extracted from a biological source such as serum. In addition, mast cells and monocytes are able to produce S1P while adipocytes produce LPA, however the main source is activated platelets. Alternatively, the phospholipid may be synthesised by procedures well known in the field of organic chemistry.

Preferably, cells that have been exposed to a phospholipid according to the

present invention are not substantially negatively affected in their ability to proliferate. Therefore, an advantage of the methods and compositions described herein is that it is possible to expand a population of hES cells without leading to a loss in pluripotency. Methods for determining the proliferative capability of a hES cell will be known by the skilled person and include detection of the cell proliferation marker PCNA as described herein. Other methods are well known and include measurement of incorporation of labelled nucelic acid precursors or assessment of colony forming ability

In another aspect the present invention provides a cell culture medium substantially free of serum, the medium including an agonist of an Edg receptor. Preferably the agonist is selected from the group consisting of S1P, LPA, PAF and SPC.

Preferably the agonist is a phospholipid, lysophospholipid or functional equivalent thereof. In a preferred form of the invention the phospholipid or lysophoholipid has a sphingosine backbone. More preferably, the lysophospholipid is a phosphorlyated amino alcohol. In a highly preferred form of the invention the lysophospholipid is sphingosine-1-phosphate or functional equivalent thereof.

The cell culture medium may be based on any of the base media known in the art useful for the growth and/or maintainance of hES cells. Such media include but are not limited to Dulbecco's Modified Eagles Medium (DMEM), KNOCKOUT-DMEM or HES medium. In a preferred form of the invention the medium is based on DMEM supplemented with insulin, transferrin and selenium.

The optimal concentration of Edg agonist in the medium may be determined by routine experimentation. However, in a preferred form of the invention the agonist is present in the medium at a concentration of from 1 μM to 20μM where the agonist is S1P. In a highly preferred form of the invention the agonist is present in the medium at a concentration of about 10μM. It would be expected that the optimum concentration will vary according to a number of

parameters including the species of agonist, the line of hES being cultured, the base medium used, and other culture conditions such as temperature, carbon dioxide concentration, and humidity.

The skilled person understands that it is often necessary to culture hES cells on feeder cells, and the present invention contemplates methods including the use of such feeder cells. The concentration of agonist may also need to be optimised according to the feeder cell line used.

In another aspect the present invention provides a human embryonic stem cell grown and/or maintained in a cell culture medium substantially free of serum, the medium including an agonist of an Edg receptor. Preferably the agonist is selected from the group including S1P, LPA, PAF, and SPC.

Preferably the agonist is a phospholipid or lysophospholipid. In a preferred form of the invention the phospholipid or lysophoholipid has a sphingosine backbone. More preferably, the lysophospholipid is a phosphorlyated amino alcohol. In a highly preferred form of the invention the lysophospholipid is sphingosine-1-phosphate or functional equivalent thereof.

Cells of the present invention will find many uses been devoted to the potential in biology and medicine. The properties of pluripotentiality and immortality are unique to ES cells and enable investigators to approach many issues in human biology and medicine for the first time. ES cells potentially can address the shortage of donor tissue for use in transplantation procedures, particularly where no alternative culture system can support growth of the required committed stem cell. However, it must be noted that almost all of the wide ranging potential applications of ES cell technology in human medicine-basic embryological research, functional genomics, growth factor and drug discovery, toxicology, and cell transplantation are based on the assumption that it will be possible to increase the proliferation and therefore grow ES cells on a large scale, to introduce genetic modifications into them, and to direct their differentiation.

The present invention also provides a method for modulating the MAP kinase pathway, the method including the step of exposing a stem cell having the pathway to an agonist of an Edg receptor. Preferably the agonist is selected from the group including S1P, LPA, PAF and SPC. Preferably the agonist is a phospholipid or lysophospholipid. More preferably, the lysophospholipid is a phosphorylated amino alcohol. In a highly preferred form of the invention the lysophospholipid is sphingosine-1-phosphate or functional equivalent thereof.

Applicants have examined whether S1P could modulate the fate of hES cells in culture. The extracellular-signal-regulated kinases (ERKs) are implicated in the regulation of critical processes such as cell proliferation or differentiation. In mouse ES cells, for example, activation of the ERK pathway is associated with induction of differentiation. Edg receptors stimulate the MAP kinase pathway. Applicants show herein that HES cells are target cells for S1P. Thus, these cells express mRNA for the Edg receptors, and the phospholipid stimulates the MAP kinases ERKs. However, in contrast to what would be predicted from studies of mouse ES cells, S1P activation of ERK kinase is correlated with an inhibition of spontaneous differentiation of HES cells.

Also provided by the present invention is a method for modulating the differentiation of a stem cell having a MAP kinase pathway and/or an endothelial gene receptor, the method including modulation of the MAP kinase pathway and/or endothelial gene receptor. Preferably the stem cell is an embryonic stem cell. More preferably the stem cell is a human embryonic stem cell.

Another aspect of the present invention is a method of treating or preventing a disorder of stem cell differentiation including administering to an animal in need thereof a composition containing an agonist of an Edg receptor. Preferably the agonist is selected from the group including S1P, LPA, PAF and SPC. Preferably the agonist is a phospholipid or lysophospholipid. More preferably the agonist is a phosphorylated amino alcohol. In a highly preferred form of the invention the agonist is sphingosine-1-phosphate. Also provided are pharmaceutical compositions including compounds of the present invention. A

skilled person will be able to provide formulations and dosage forms of the agonist. Furthermore, the optimum dosage for a given clinical situation could be determined by routine experimentation.

The invention will now be more fully described with reference to the following non-limiting Examples.

EXAMPLES

The following materials and methods were used in the Examples below.

Reagents

S1P was obtained from Biomol (Plymouth Meeting, PA, USA) and were dissolved in methanol. Freshly prepared dilutions of all agonists were made in water containing 0.1% fatty acid-free bovine serum albumin (BSA) (Sigma). Protease, sodium orthovanadate and U0126 were from Sigma. was from Calbiochem (San Diego, CA, USA). *Pertussis* Toxin (PTX) was from List Biological Laboratories (Campbell, CA, USA). GCTM-2, Oct-4, PCNA, Hoechst-33342

Cell culture

HES-3 cells were cultured as previously described Human stem cells were grown on MMC treated fibroblasts' feeder layer. Fibroblasts were plated on gelatine treated dishes. A combination of human and mouse derived fibroblasts were used at a density of approximately 25,000 and 70,000 cells per cm2 respectively. The fibroblasts were plated up to 48 hours before culture of the stem cells. Mouse fibroblasts only could also support the growth of the stem cells. However, while human fibroblasts could also support stem cells, they created an uneven and unstable feeder layer. Therefore, the human fibroblasts were combined with the mouse fibroblasts to augment and achieve better support of growth and prevention of differentiation.

The medium that was used for the growth of human stem was DMEM (GIBCO, without sodium pyruvate, with glucose 4500mg/L) supplemented with 20% FBS

(Hyclone, Utah) (2-mercaptoethanol - 0.1mM (GIBCO), Non Essential Amino Acids - NEAA 1% (GIBCO), glutamine 2mM.(GIBCO), penicillin 50u/ml, and streptomycin 50mg/ml (GIBCO)

For direct observation, HES-3 cells were coated into 12-well plates (3 colonies per well), with or without mouse embryonic feeders (MEFs). The day following the plating, cells were incubated with the different agents in serum free medium containing insulin, transferring and selenium. Media was changed the 2nd day and then every 2 days.

For immunostaining, HES-3 cells were coated on chamber slides after mechanical dissociation, in order to obtain a monolayer culture. The day following the plating, cells were incubated with the different agents in a media depleted in serum. Media was changed the 2nd day and the cells were fixed 4 days after the first treatment.

For immunoblot analysis, cells were transferred into 24 well plates (8 colonies per well) without MEFs, and 24 hr later, were grown in the absence of serum for 18 hrs.

In some experiments, cells were pre-treated for 1 hr with U0126 (30 μM) or for 18 hrs with PTX (100 μg/ml).

RT-PCR experiments

Cells were washed with PBS and HES colonies were removed by treatment with protease. Purified mRNA was extracted from HES cultures using Dynabeads® Oligo $(dT)_{25}$ (Dynal, Oslo, Norway), according to the supplier's instruction. RT was performed using superscript™ II Rnase H$^-$ Reverse Transcriptase (Invitrogen, Life technologies), according to the supplier's protocol. After cooling on ice, the cDNA samples were amplified by PCR with sense and antigens primers (synthesis performed by Sigma Genosys, Castle Hill, Australia) designed for the specific detection of human Edg-1, Edg-2, Edg-3, Edg-4, Edg-5, Edg-6, Edg-7 and Edg-8 DNA target sequences. The primers

used for Edg-1, Edg-3, Edg-5, Edg-6 and Edg-8 were previously designed by Hornuss *et al.* (2001) [1]. These primer pairs were : 5'-CCACAACGGGAGCAATAACT-3' (sense) and 5'-GTAAATGATGGGGTTGGTGC-3' (antigens) (expected PCR product: 480 bp) for Edg-1 ; 5'-TCAGGGAGGGCAGTATGTTC-3' (sense) and 5'-CTGAGCCTTGAAGAGGATGG-3' (antisense) (505 bp) for Edg-3 ; 5'-CCAATACCTTGCTCTCTCTGGC-3' (sense) and 5'-CAGAAGGAGGATGCTGAAGG-3' (antisense) (502 bp) for Edg-5 ; 5'-CGGCTCATTGTTCTGCACTA-3' (sense) and 5'-GATCATCAGCACCGTCTTCA-3' (antisense) (701 bp) for Edg-6 ; and 5'-TTCTGATACCAGAGTCCGGG-3' (sense) and 5'-CAAGGCCTACGTGCTCTTCT-3' (antisense) (460 bp) for Edg-8 . For Edg-2 and Edg-4, the primer pairs designed by Goetzl *et al.* (1999) were used: 5'-GCTCCACACACGGATGAGCAACC-3' (sens) and 5'-GTGGTCATTGCTGTGAACTCCAGC-3' (antisense) (621 bp) for Edg-2, and 5'-AGCTGCACAGCCGCCTGCCCCGT-3' (sense) and 5'-TGCTGTGCCATGCCAGACCTTGTC-3' (antisense) (775 bp) for Edg-4. For Edg-7, the primer pairs designed by Goetlz *et al.* (2000) were used: 5'-CCATAGCAACCTGACCAAAAAGAG-3' (sense) and 5'-TCCTTGTAGGAGTAGATGATGGGG-3' (antisense) (482 bp). PCR experiments were performed in a mixture (25 µl) containing 0.25 units of Taq DNA polymerase (Biotech International Ltd, Perth, WA, Australia) and 2 µM of each primer in a buffer including 67 mM Tris-HCl, pH 8.8, 1.5 mM $MgCl_2$, 16.6 mM $[NH_4]_2SO_4$, 0.45% Triton X-100, 0.25 mM of each dATP, dGTP, dCTP, dTTP. Absence of contaminating genomic DNA was confirmed by control reactions with mRNA that had not been treated with reverse transcriptase. PCR experiments were run with the following steps: initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 30 sec, annealing at 52°C (Edg-1, Edg-3, Edg-5, Edg-6, Edg-8) or 56°C (Edg-2, Edg-4, Edg-7) for 2 min, extension at 74°C for 2 min, and a final extension at 74°C for 7 min. The specific amplified DNA fragments were purified by electrophoresis on 1.5 % (w/v) agarose gel, stained with ethidium bromide and photographed. The amplicons were purified and sequenced.

Immunofluorescence

Cells were washed in PBS, fixed with MeOH, and immunostaining was performed, using the specific stem cell marker antibody GCTM-2, and the specific cell proliferation marker PCNA. Cells were then washed and the nucleuses were stained with Hoechst-33342 (1μg/ml). Slides were mounted and then observed by fluorescent microscopy. Cells were then counted in order to determine the ratio of proliferating stem cells within the overall population.

Western blot analysis

HES3 cells were lysed following removal of the supernatants by addition of a reducing loading buffer (2% SDS, 62.5 mM Tris pH 6.8, 0.1 M DTT, 0.01% bromophenol blue) containing 1 mM sodium orthovanadate. Samples were boiled for 10 min and centrifuged at 13000g for 15 min, and protein lysates (approx. 80 μg) were separated by SDS-polyacrylamide gel electrophoresis (10% polyacrylamide, w/v). Proteins were transferred to nitrocellulose (Hybond-ECL, Amersham) and immunoblotting was carried out with rabbit polyclonal anti-active mitogen-activated protein (MAPK) antibodies raised against a dually phosphorylated MAPK peptide (Promega, Madison, WI, USA). Peroxidase-coupled secondary antibody (Dako) was detected by exposure of autoradiographic films in the presence of a chemiluminescent detection reagent (ECL, Amersham). Stripping of antibodies was achieved by incubating the membrane during 30 min at 50°C in a buffer containing 100 mM βmercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.7. The membrane was then reprobing with rabbit polyclonal anti-ERK1/2 antibodies (Promega), and then with peroxidase-coupled secondary antibodies (Dako).

Blots probed with either rabbit polyclonal anti-active p38 (Promega) or rabbit polyclonal anti-active JNK (Promega) or mouse polyclonal GCTM-2 antibodies were also performed, using the same procedure as described above.

Protein quantification

HES3 cells were lysed and their quantity was determined by using a

colorimetric assay based on the Bradford dye-binding test (Bio-Rad Laboratories, Regents Park, NSW, Australia).

Each set of experiments was performed at least 3 times (*n* refers to number of independent experiments performed on different cell cultures).

EXAMPLE 1: Edg mRNAs are expressed by HES cells

The results presented in Figure 1A indicate that HES cells expressed mRNA transcripts for the three S1P receptors : Edg-1, Edg-3 and Edg-5 while these cells do not seem to express mRNA for Edg-6 and Edg-8 (data not shown). Moreover, HES cells express mRNA transcripts for each of LPA receptors : Edg-2, Edg-4 and Edg-7 (Figure 1B). The nucleotide sequences of all purified PCR products were analysed and revealed to be identical to the corresponding regions in the human receptor genes.

EXAMPLE 2: S1P prevents HES cells from spontaneous differentiation

Applicants next determined whether S1P and/or LPA could modulate the fate of HES cells. When HES cells were grown on MEFs, in a culture media depleted in serum, they spontaneously differentiated. As shown in Figure 2, after 8 days in such conditions, HES cells colonies contained enlarged flattened cells which formed cystic structures (Figure 2A, 2B). Even after 12 days, LPA (up to 50 μM) did not seem to affect the growth of the colonies (data not shown). In presence of S1P (10 μM, 8 days), the colonies were more compact and less differentiated than in the control condition (Figure 2C). This effect of S1P was more obvious after 12 days of treatment (Figure 2D, 2E). The inhibitory effect of S1P on cell differentiation and the lack of effect of LPA were also observed when HES cells were grown without MEFs, suggesting that S1P did not directly act on the feeder cells (n=3, data not shown).

In order to understand and quantify the effect of S1P on the spontaneous differentiation of HES cells, double immunostaining experiments were carried out. For that purpose, Applicants used two specific antibodies, one as a stem

cell marker, GCTM-2, and one for proliferation, PCNA, a marker that is only expressed during the S phase of the cell cycle, in order to determine the ratio of proliferating stem cells (Figure 3). After 4 days in a media without serum, most of the control cells were differentiated (Figures 3A, 3C and 3E), as revealed by the fact that only 16 % of the cells still expressed GCTM-2 (Figure 4A). By contrast, when S1P (10 μM) was added to the media, 68 % of the cells were GCTM-2 positive, suggesting that most of the cells remained stem cells (Figures 3B, 3D, 3F and 4B). Within these cell populations, a large part expressed PCNA, suggesting that most of these stem cells still proliferated (Figures 3G and 3H). However, no marked difference in the proliferating rate of HES cells between the control cells and the ones treated with S1P were observed (Figure 4). Altogether, these data suggest that S1P mostly acts on the differentiation of HES cells observed in absence of serum rather then acts on the proliferating state of HES cells.

EXAMPLE 3: S1P activate ERKs in HES cells

Because the MAP kinases ERKs have often been implicated in cell proliferation and differentiation, the effects of S1P on the activation of the ERKs were then investigated. After 5 min, S1P stimulated the phosphorylation of ERKs in HES cells (Figure 4), an effect that was totally inhibited in presence of the MEK inhibitor U0126 (30 μM) (Figure 4A). S1P stimulated ERKs for at least 60 min and in a concentration dependant manner (Figure 4B, 4C).

These results show clearly that treatment of human ES cells with S1P results in inhibition of spontaneous differentiation. S1P is a major component of serum, and is therefore likely to account for much of the beneficial effect of calf serum in human ES cultures.

REFERENCES

Hornuss, C., et al., Human and rat alveolar macrophages express multiple EDG receptors. Eur J Pharmacol, 2001. **429**(1-3): p. 303-8.

Goetzl, E.J., et al., Dual mechanisms for lysophospholipid induction of proliferation of human breast carcinoma cells. Cancer Res, 1999. **59**(18): p. 4732-7.

Goetzl, E.J., Y. Kong, and J.K. Voice, Cutting edge: differential constitutive expression of functional receptors for lysophosphatidic acid by human blood lymphocytes. J Immunol, 2000. **164**(10): p. 4996-9.

Finally it is to be understood that various other modifications and/or alterations may be made without departing from the spirit of the present invention as outlined herein.

DATED: 7 June, 2002

PHILLIPS ORMONDE & FITZPATRICK

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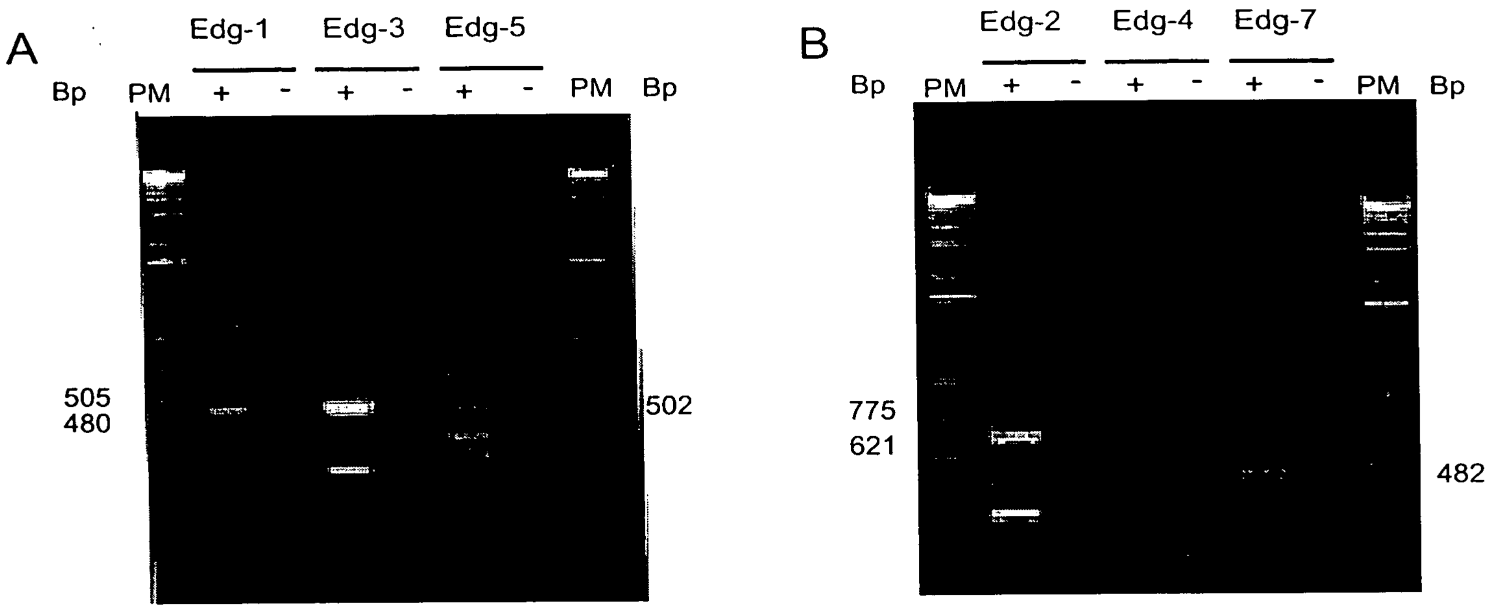
David B Fitzpatrick

ES CELL INTERNATIONAL PTE LTD


A
Edg-1
Edg-3
Edg-5
Bp
PM
+
-
+
-
+
-
PM
Bp
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Edg-2
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775
621
482

FIGURE 1

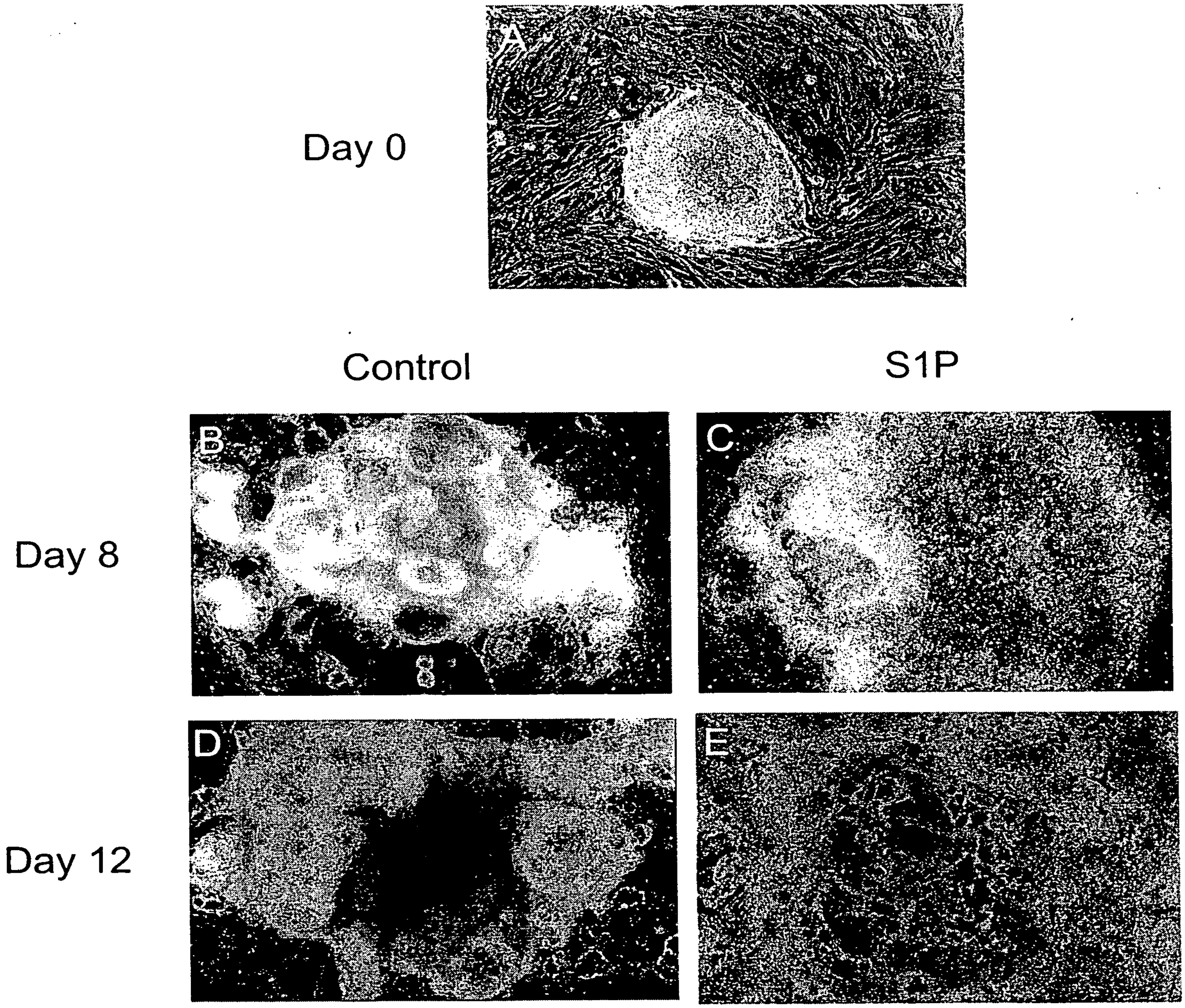

FIGURE 2

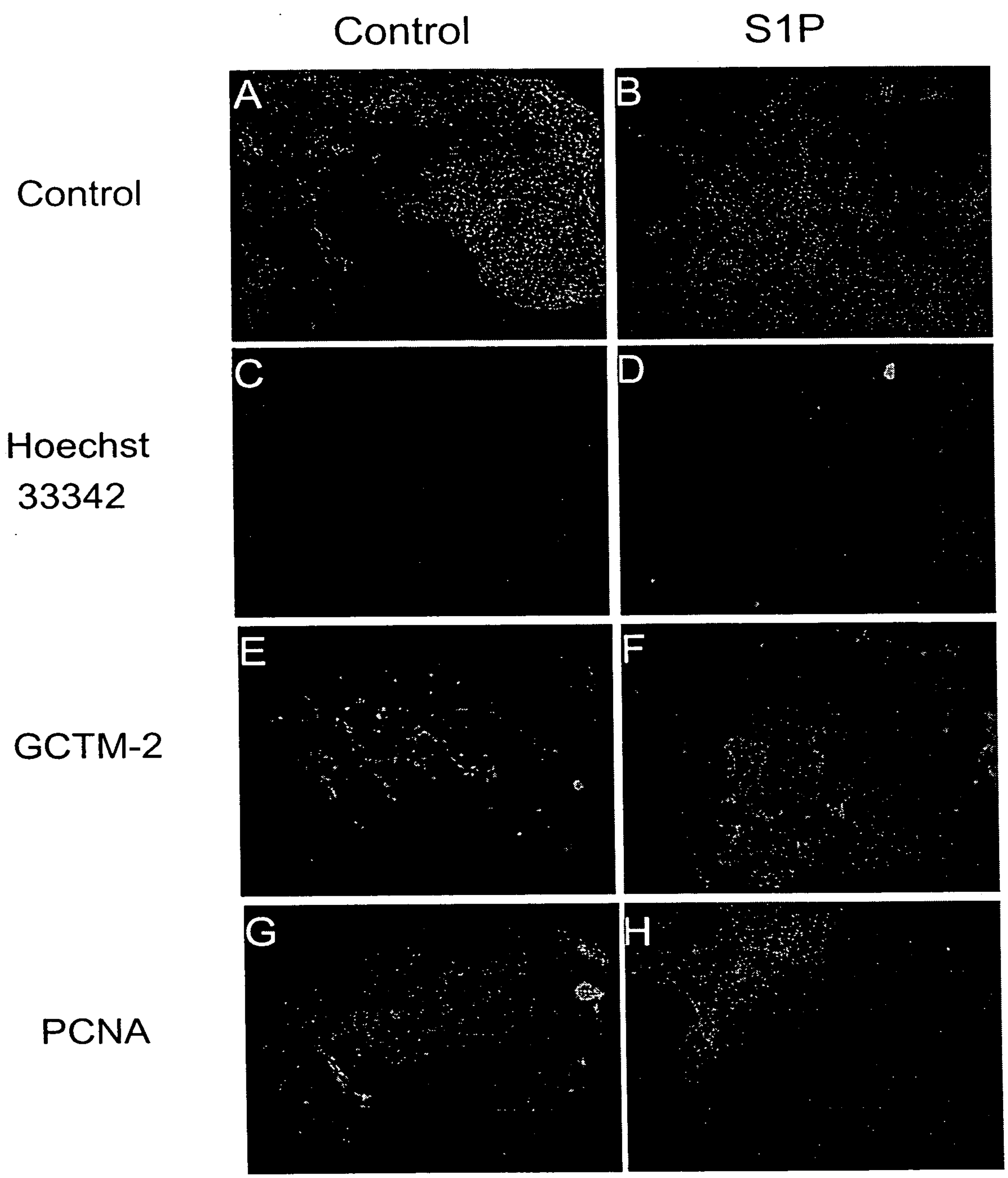

FIGURE 3

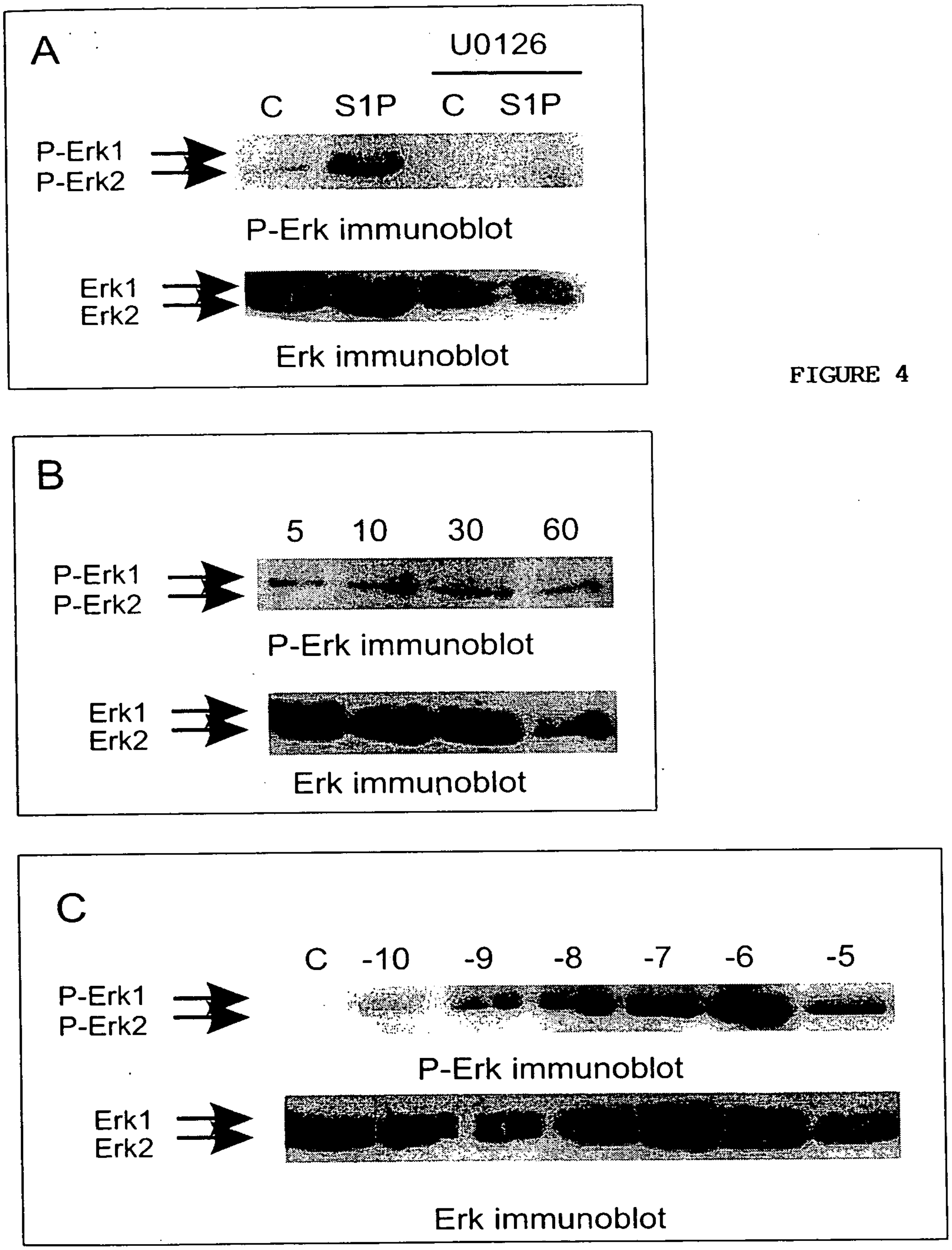
A
U0126
C
S1P
C
S1P
P-Erk1
P-Erk2
P-Erk immunoblot
Erk1
Erk2
Erk immunoblot
B
5
10
30
60
P-Erk1
P-Erk2
P-Erk immunoblot
Erk1
Erk2
Erk immunoblot
C
C
-10
-9
-8
-7
-6
-5
P-Erk1
P-Erk2
P-Erk immunoblot
Erk1
Erk2
Erk immunoblot

FIGURE 4